Biomedical Reviews 1996; 6: 75-81
ISSN 1310-392X

AN OVERVIEW OF THE REGENERATION OF SKELETAL MUSCLE

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SUMMARY

• It has only recently been acknowledged widely that mature skeletal muscle has the ability to regenerate, although reports on this phenomenon have existed in the research literature some 40 years. The regenerative events in skeletal muscle include: phagocytosis of cellular and connective tissue debris; revascularisation of the lesion; the proliferation of myogenic precursor cells; their differentiation into myoblasts; myoblasts fusion into myotubes; followed by the reestablishment of the nerve supply, and the maturation ofmyotubes into muscle fibres. The key cell in skeletal muscle regeneration is the satellite cell, which is a reserve myogenic cell situated in between the muscle fibre sarcolemma and its adjacent external lamina. These cells can only by identified by electron microscopy, which makes them very difficult to investigate in detail or quantitatively. However, there is a substantial body of research literature on satellite cells and relevant aspects of their activity are summarized in this review. Satellite cells provide the source of myogenic precursor cells in the regeneration of skeletal muscle, therefore, any factor which stimulates the proliferative activity of satellite cells is very important in enhancing skeletal muscle regeneration. The cellular events in regenerating skeletal muscle closely resemble those which occur in the process of developmental myogenesis, and references to these similarities and differences are briefly reviewed.

INTRODUCTION

• The regeneration of adult skeletal muscle following all types of injury consists of the following events: necrosis; phagocytosis; revascularisation; proliferation of myogenic precursor cells (MFC); differentiation of MFC into myoblasts and their fusion into myotubes; reinnervation and maturation of the new muscle fibres. The following review describes the regenerative process of mature skeletal muscle.

THE PATTERN OF REGENERATION

- **Necrosis of muscle fibres**

Following injury of skeletal muscle, the first recognizable response of the damaged myofibres is necrosis. Necrotic muscle fibres can be described as having lost the structural attributes of living muscle (1), and this necrosis is characterized by several histological changes. Electron and light microscopic studies show: a pyknosis and eventual death of myonuclei, breakdown of the complex contractive protein arrangement, destruction of myofibrillar material (initially Z bands dissolve), vesiculation of mitochondrial crystal membranes, accumulation of vesicles within the sarcoplasm, disappearance of glycogen and fibrosomes, and dissolution of the sarcolemma (2-5).

While myofibres become necrotic due to injury, the external (pericellular, "basal") lamina of each myofibre appears initially to survive most forms of injury, and it subsequently plays an important role in the formation of new muscle fibres (3, 6). Additionally, it appears that satellite cells (described below) survive injury. In fact, they are remarkably resistant to the ischemia associated with even most severe injuries, including transplantation of whole muscle (7-12), while they remain viable, in contrast to the myonuclei of associated myofibres, which become pyknotic and die (3, 13-16).

- **Phagocytosis**

The next stage of the regenerative process is phagocytosis of the necrotic debries. Polymorphonuclear leukocytes, and then macrophages appear to initiate phagocytosis of damaged skeletal muscle (2,3, 8, 17), macrophages appearing to be the primary phagocytic cell responsible for remodeling of the necrotic tissue (3,8,17-19). In cases of extensive injury to the vascular supply, phagocytes appear in the periphery of the muscle just before the appearance of ingrowing blood vessels (2,3, 20), and may play a role in assisting revascularisation indeed.

- **Revascularisation**

Revascularisation is an essential process in the regeneration of skeletal muscle, particularly following severe injury: in the absence of blood vessels skeletal muscle fibres will not regenerate, rather they become necrotic and are replaced with fibrous scar tissue (21). Revascularisation of regenerating skeletal muscle was first investigated *via* a series of experiments in which the vessels in whole minced skeletal muscle graft were perfused with Indian ink (22). In intact whole muscle grafts where the entire vascular supply to the graft is similarly severed, revascularisation is relatively slow as blood vessels must grow into the muscle graft from surrounding host tissues (7) through physical barriers such as the epimysium. In the case of "large" graft, considered by Carlson (23) to include graft greater than 6 g, this can mean that the ischemic central core of the graft, does not regenerate. It instead becomes fibrotic and filled with connective tissue (4,24), possibly because ischemia and low oxygen tension promote the proliferation of fibroblasts (25).

By the time new blood vessels grow into damaged skeletal muscle the original vasculature has, along with the myofibres, started to become necrotic (4,26). Thus new blood vessels must grow through existing necrotic fibres to reach the centre of the lesion (3, 27, 28). Invading sinusoidal vessels grow into the area from adjacent tissues by branching around surviving muscle fibres. From these vessels the capillaries sprout that later differentiate into arterioles and venules and these represent the new vascular system of the regenerating muscle (28). The original vascular structures are thought to play a limited role in revascularisation. Hansen-Smith *et al* (28) observed that preexisting vessels and external laminae within the area of damaged muscle were only utilized by ingrowing endothelial cells at the later stages of regeneration. However, in a study which used small grafts of bundles of muscle fibres inserted into hamster cheek pouches (24), original blood vessels in the graft were found to survive the transplantation procedure and they sprouted out towards the vessels invading from the adjacent tissues in the cheek pouch, eventually anastomosing with them. These differences in the role of the preexisting vasculature in revascularisation may be a function of the size of grafts used in the different studies. Treating regenerating muscle in small experimental transplants with vasoactive agents, such as clenbuterol and isoprenaline, which accelerate revascularisation improves regeneration (11,29). However, in other studies performed on crushed muscle, the use of other agents, such as basic fibroblast growth factor and eurcamide, an omental extract, to in-' duce blood vessel growth has not enhanced regeneration (30).

- **The proliferation of myogenic precursor cells**

- *Satellite cells*

Satellite cells are undifferentiated mononucleated "reserve" muscle cells so named "satellite" cells, because they occupy a satellite position between the external lamina of the muscle fibre and the sarcolemma. Satellite cells were first described in 1961 by Alexander Mauro in an electron microscopic study of the peripheral region of the skeletal muscle fibre of the frog (31). Satellite cells have been described within the skeletal muscle fibres of almost all vertebrates (32). While they are typically reported as appearing fusiform (33) with scant cytoplasm and few distinctive organelles (7, 34), their appearance does vary considerably, particularly when in different states of activity. In the active state, satellite cells have more abundant cytoplasm filled with organelles, while, in the inactive or quiescent state, satellite cells typically have a heterochromatic nucleus and less apparent nucleoli (32).

As satellite cells are positioned within 15-60 nm of the external lamina (32), their position outside the sarcolemma can only be resolved using the electron microscope (8, 17, 31). At the light microscopic level, satellite cells which have scant cytoplasm are indistinguishable from nearby myonuclei which are of similar size. Unlike myonuclei (which are post-mitotic) lying within the muscle fibre, satellite cells retain the capacity to reenter the mitotic cycle and divide at a later time, such as in response to injury. Satellite cells of old muscle still have the capacity to replicate, as has been shown in cultured muscle (35) and in regenerating muscle (36).

• *Satellite cells as the source ofmyogenic precursor cells in regenerating muscle*

There is strong evidence for the hypothesis that the MFC responsible for regenerating muscle are derived from satellite cells (7, 8, 17, 31, 32, 37-41). For example, it has been demonstrated by tritiated thymidine labelling that satellite cells undergo mitosis to produce MFC which fuse and form myotubes *in vivo* (8, 17). Also, studies *in vitro* show that MFC derived from satellite cells becoming directly incorporated into myotubes (42, 43).

Replication of MFC appears to be related to the arrival of phagocytes in the periphery of regenerating skeletal muscle grafts (3, 8, 44). Many satellite cells proliferate beneath the surviving external laminae of their associated muscle fibres (3,6,7). In response to trauma in mature muscle, satellite cells shift from a morphologically quiescent state of the cell cycle (G_0) to an activated state (G,) (34). When satellite cells become activated they develop more cytoplasm, increased number of organelles, and large euchromatic nuclei with prominent nucleoli (3, 4, 45). The stimulus for the activation of satellite cells and their shift into the G, phase of the cell cycle is not clear, and this is the subject of ongoing research in our laboratory. Ingrowing blood vessels and associated phagocytotic cells are thought to be closely associated with the activation of satellite cells in regenerating skeletal muscle (3, 4, 28, 44, 46, 47), possibly through the release of stimulating factors. Using autoradiographic techniques shows that MFC in whole intact muscle graft in mice start DNA synthesis at 48 hours after grafting (44). Since the entry of phagocytes and ingrowing blood vessels are not seen until 72 hours after grafting (20) this suggests that activating factors such as nutrients and various mitogens must be diffusing inwards ahead of the phagocytic cells to reach surviving satellite cells within the graft (20). Other possible influences on the activation of satellite cells based on studies performed *in vitro* and *in vivo,* include molecules released following denervation and necrosis (43). However, due to the complex nature and interaction of regulatory factors the precise agents responsible for activating satellite cells *in vivo,* are not known yet (48, 49).

The time of onset of proliferation in MFC associated with injury varies with the severity of injury. In skeletal muscle traumatized by cut and crush injuries, MFC begin to proliferate by 24-30 hours, and complete their proliferative activity by about 5 days (50), whereas replication finishes far later in minced muscle isografts (51) and does not even begin until 48 hours in the transplanted *extensor digitorum longus* muscle model (44). The precise time of onset of MFC replication can also vary between different mouse strains (36). Within the minced muscle transplants of SJL/J mice begin to proliferate earlier (24 hr) than within the minced transplants of BALB/c mice (30-36 hr) (51). Thus it appears that the onset of MFC proliferation is influenced not only by the severity of the injury (49), but also by the strains of animals being investigated.

• *Satellite cell migration*

Several studies have presented evidence supporting the migratory ability of satellite cells in association with injury. In early regenerating muscle, satellite cells appeared to be moving along the muscle fibre but they maintained their position between the external lamina and the sarcolemma (52), and migrating satellite cells have been shown to travel within a muscle from undamaged areas to the site of injury (53). Furthermore, the migration of satellite cells into the interstitial space was reported in denervated rat tibialis anterior muscle and mouse lumbrical muscles (45). Similar migration was also observed from single muscle fibers *in vitro* (54). In more recent studies, injected MFC are attracted into and migrate to the site of injury in rat and quail muscles (55). There is also evidence of satellite cells migrating into grafted (injured) muscle from surrounding (host) tissue (56-59), although in rats it appears that migration (into the graft from the host) only occurs following pre-injury to the epimysial connective tissue barrier separating the graft and host muscles (60).

• Differentiation of myogenic precursor cellsinto myoblasts and their fusion into myotubes

The proliferation of MFC, their differentiation into myoblasts and fusion to form myotubes essentially recapitulates the myogenic events mat occur in the embryo. In regenerating muscles, differentiating myoblasts can be recognized microscopically as cells with open-faced nuclei and basophilic cytoplasm containing large quantities of free ribosomes, thick and thin filaments, primitive Z bodies and assembling myofibrils (2,4,7). In preparation for fusion, myoblasts often align and cluster along the inner surface of the tube of the persisting external lamina, in what has been described as "basophilic cuffs" (2,7), with parts of the plasma membrane in contact with the external lamina (3, 6, 8, 17, 61). Myoblasts then fuse to form multinucleated myotubes characterized by chains of central nuclei (4); later to form the typical syncytial arrangements of skeletal muscle fibres.

Pattern of myotube formation

The process of myotube formation that occurs in regenerating adult muscle differs in some respects from that seen in the embryo. In regenerating adult muscle, the external lamina which usually survives injury, directs the alignment and orientation of the newly formed myotubes (40), whereas this is not present at the time when primary myotubes form in the embryo. The myotube formation patterns in regenerating muscle is con-

sidered to be biphasic. However, as in the embryo (3, 62) and in adult regenerating muscle grafts, the number of myotubes formed within one external lamina can vary between one and seven (61, 63). As part of the process of myofibre maturation in the embryo the myonuclei within myotubes migrate peripherally (7), whereas in regenerated skeletal muscle myofibres tend to retain their centrally located nuclei (3, 64).

- *External lamina*

The remnants of external laminae belonging to the original myofibres of a muscle graft survive transplantation, but not so after some other forms of injury. These laminae act as a scaffolding for the maturing muscle fibres (6). Once the immature myotubes have formed in muscle grafts, new external laminae begin to form along portions of the plasma membrane surrounding the myotubes (6). This means that when examined ultrastructurally, myotubes may have two surrounding external laminae, although eventually they are encased by just one (6,62).

- **Reinnervation**

In the reinnervation of injured skeletal muscle, most regenerating axons are attracted back to the original synaptic sites (28) on the surviving external laminae (23). However in some cases, such as after minced muscle transplantation, new neuromuscular connections derived from ingrowing axons can form in "ectopic" locations (65). New nerve fibres enter minced muscle grafts in the second week of regeneration, and then progressively increase in number (66,67). In intact whole muscle grafts the extent of regeneration is such that by the third week after surgery muscle transplants exhibit functional neuromuscular transmission (23) and this eventually reaches 60% of normal, pretransplantation levels (15). Reinnervation is essential for the maturation of regenerating myofibres, including the development of cross striations and the differentiation into various fibre types. Denervation studies show that without innervation regenerated muscle fibres remain functionally and histochemically immature (4,64) and eventually atrophy (3). The possible involvement of neuroimmunological mechanisms (68) in the regenerative process of skeletal muscle may be examined.

CONCLUSION

• An increasing amount is becoming known about the events involved in the regeneration of mature skeletal muscle. However, there is still a great deal to be learned, especially about the factors which enhance the myogenesis and the fusion of myoblasts into myotubes. The genetic phenomena which initiate myogenesis are the subject of intense interest, as is the role of extracellular matrix in the activity of myogenic cells and their intercellular relationships, especially during fusion of myoblasts into myotubes. The muscular satellite cell is not completely understood yet, even though it has been investigated in detail for at least 30 years. When some of these mechanisms are more clearly understood, we can hope to improve the outcome of surgical procedures to improve muscle repair and fimction, and to reverse some of the tragic sequelae of the crippling muscular dystophies.

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Received 28 May 1996
Accepted 3 July 1996

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